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Aberrant Glycosylation of the IgA1 molecule in IgA Nephropathy

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Abstract

IgA nephropathy, the most common primary glomerulonephritis in the world and a frequent cause of end-stage renal disease, is characterized by typical mesangial deposits of IgA1, as first described by Berger and Hinglais in 1968. Since then, it was discovered that aberrant IgA1 *O*-glycosylation is involved in disease pathogenesis. Progress in glycomic, genomic, clinical, analytical, and biochemical studies revealed autoimmune features of IgA nephropathy. The autoimmune character of the disease is explained by a multi-hit pathogenesis model, wherein overproduction of aberrantly glycosylated IgA1, galactose-deficient in some *O*-glycans, by IgA1-secreting cells leads to elevated levels of circulatory galactose-deficient IgA1. These glycoforms induce production of autoantibodies that subsequently bind hinge-region galactose-deficient IgA1 molecules, resulting in the formation of nephritogenic immune complexes. Some of these complexes deposit in the kidney, activate mesangial cells, and incite glomerular injury. Thus, galactose-deficient IgA1 is central to the disease process. In this chapter, we review studies concerning IgA1 *O*-glycosylation that contributed to the current understanding of the role of IgA1 in pathogenesis of IgA nephropathy.

Summary

IgAN was first described by Berger and Hinglais in 1968 as a mesangioproliferative glomerular disease with typical mesangial deposits of IgA1 and IgG. Since then, IgAN has been recognized as the most common primary glomerulonephritis in the world. Progress in the studies of IgA1 glycosylation indicated an association of aberrant IgA1 *O*-glycosylation, Gal deficiency, with disease pathogenesis. More recently, autoimmune features of IgAN have been recognized, as explained by a multi-hit pathogenesis model. Specifically, aberrantly glycosylated IgA1, an

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increased number of Gal-deficient *O*-glycans in the hinge region, is overproduced by IgA1-secreting cells, resulting in elevated levels of circulatory Gal-deficient IgA1. These glycoforms induce production of autoantibodies that subsequently bind hinge-region Gal-deficient IgA1 molecules, resulting in the formation of circulating immune complexes. Some of these complexes deposit in the mesangium, activate resident cells, and incite renal injury. Thus, Gal-deficient IgA1 is central to the disease process. In spite of all the accumulated knowledge on IgA1 *O*-glycosylation, more work remains to be done to fully understand the pathogenesis of IgAN at molecular and cellular levels to and design effective disease-specific treatments.

Keywords

O-glycans; IgA1; signaling; autoantibody

Introduction

IgA nephropathy (IgAN) is the most common primary glomerulonephritis in the world and a frequent cause of end-stage renal disease.¹ The diagnosis is established by the presence of IgA as the dominant or co-dominant immunoglobulin in the glomerular mesangium on examination of renal tissue by routine immunofluorescence microscopy or immunohistochemistry.^{2,3} The IgA is exclusively of the IgA1 subclass⁴ and is galactose-deficient compared with the carbohydrate composition of serum IgA1 in healthy individuals.^{5,6} IgG, IgM, or both may also be present. In a substantial minority of patients, IgA is the sole immunoglobulin detected. A potential clinical significance in the variation of the immunoglobulin co-deposits is underscored by a study that found that localization of glomerular IgA in the mesangium and capillary walls and the presence of IgG in the mesangium correlated with higher mesangial and endocapillary cellularity scores.⁷ Complement component C3 frequently co-localizes with the IgA, consistent with activation of complement via the alternative pathway. The presence of mannan-binding lectin with IgA, C3, and C4d is consistent with activation of the lectin pathway in some patients. C1q is absent or minimally present in most cases. Confocal microscopy shows co-localization of immune proteins, consistent with the presence of immune complexes.⁸ By light microscopy, mesangial cellular proliferation and expansion of the extracellular matrix are characteristic findings. In 2009, the Oxford classification standardized the description of light-microscopy features of IgAN⁹ and three, mesangial hypercellularity (M), segmental glomerulosclerosis (S), and moderate to severe tubular atrophy / interstitial fibrosis (T), independently correlated with a poor clinical outcome (50% decline in estimated glomerular filtration rate or end-stage renal disease).¹⁰ Endocapillary hypercellularity (E) was predictive of a poor outcome in patients who did not receive immunosuppression but lost its predictive value when immunosuppression was given, providing indirect evidence that endocapillary lesions are responsive to immunosuppressive therapy.¹⁰ Additional scoring for presence of cellular/fibrocellular crescents has been recently added.¹¹ Crescents in less than one fourth of glomeruli identified patients at increased risk for a poor outcome without immunosuppression while crescents in more than a fourth of glomeruli identified patients at increased risk for poor outcome even with immunosuppressive therapy.¹¹ Electron microscopy typically shows electron-dense deposits consistent with immune complexes in

the mesangial and paramesangial areas, sometimes with occasional subepithelial or subendothelial deposits. Electron microscopy also may reveal effacement of the foot processes of podocytes in association with substantial proteinuria.

IgAN often first manifests in children, adolescents, and young adults as painless macroscopic hematuria concurrent with an infection of the upper respiratory tract or digestive system. In adults older than 35 years, asymptomatic proteinuria, microscopic hematuria, and hypertension are typical clinical presentations. Disease incidence varies greatly by geographical location.¹² IgAN accounts for up to 40% of native-kidney biopsies in eastern Asia, versus 5–20% in Europe and the United States and less than 5% in central Africa.¹² In some regions of the southeastern United States, African-Americans may be affected at the same rate as whites.^{13–15} IgAN usually affects more males than females (2–3:1) in the United States and Europe but affects both genders equally in eastern Asia. Some geographic and gender variability in disease frequency may be due to differences in access to medical care or criteria for performing a renal biopsy. Subclinical disease is more common than generally appreciated. In a report from Singapore, glomerular IgA was found in eight of 200 (4%) consecutive autopsies of persons who had died of traumatic injuries without clinical history of renal disease or major non-renal disease discovered postmortem; IgG or IgM was detected in the mesangium in four and two cases, respectively.¹⁶ The one case with mesangial cell proliferation had deposits of IgG. A study in Japan demonstrated mesangial IgA deposits in 82 of 510 renal allografts (16%) at implantation, of which 19 showed proliferative lesions by light microscopy.¹⁷

Another basis for the diverse variation of disease incidence is the impact of genetically determined factors on the mechanisms of disease. Fifteen loci have been associated with IgAN in genome-wide association studies and the prevalence of these loci correlated with the frequency of the disease.^{18,19} Several of these loci encode proteins that modify activation of the alternative pathway of complement²⁰ or the enzymatic *O*-glycosylation of IgA1.²¹ These findings indicate that genetically determined factors influence the development or clinical expression of IgAN even in patients without familial IgAN.

IgA1 structure and glycosylation

Humans have two IgA subclasses, IgA1 and IgA2. Both subclasses are quite similar, but differ in several important aspects. Notably, IgA1, unlike IgA2, has a unique hinge-region segment connecting the C_α1 and C_α2 domains of the heavy chain that is the site of attachment of clustered *O*-glycans (Figure 1).^{22–24} IgA1 hinge region consists of two octapeptide repeats rich in Ser, Thr, and Pro residues and has nine potential *O*-glycosylation sites. Usually, only three to six of these sites are *O*-glycosylated (Figure 1, bottom).^{25–28} *O*-glycans of serum IgA1 of healthy individuals are usually core 1 glycans, *i.e.*, disaccharides consisting of *N*-acetylgalactosamine (GalNAc) attached to a Ser/Thr residue and galactose (Gal) linked to GalNAc through a β1,3 glycosidic bond. GalNAc-Gal disaccharide may be sialylated on GalNAc, Gal, or both sugars.^{29,30} *N*-acetylneuraminic acid can be attached to GalNAc through an α2,6-linkage and/or to Gal by an α2,3-linkage. IgA1 also has two *N*-glycosylation sites on each heavy chain containing complex glycans.^{23,30–33}

Humans produce about 70 mg of IgA per kg of body weight daily.³⁴ In the circulation, IgA is predominantly monomeric and of the IgA1 subclass; the usual serum concentration is approximately 2 mg/ml. Monomeric human IgA1 has two heavy chains and two light chains connected by disulphidic bridges³⁵ (Figure 1). Monomers of IgA1 (as well as IgA2) can be covalently associated with J chain during production in IgA-secreting cells,³⁶ resulting in polymeric IgA, usually dimers or higher oligomers.

Most IgA is synthesized in mucosal tissues in the polymeric form that is transported by a polymeric immunoglobulin receptor (pIgR)-mediated pathway into the mucosal secretions (secretory IgA that has attached secretory component, a fragment of pIgR); only a small percentage of polymeric IgA enters the circulation.³⁷ IgA in the circulation is produced mainly in the bone marrow and to a lesser extent in the spleen and lymph nodes. A contribution by tonsillar IgA1-producing cells to serum IgA1 has been considered to play a role in IgAN.^{38–41}

Analysis of IgA glycosylation

Lectins and monosaccharide compositional analysis—First insights into glycosylation of IgA1 and IgA2 were based on monosaccharide compositional analyses of IgA myeloma proteins by a then new technique, gas-liquid chromatography.^{23,24,31} This technique uses acid hydrolysis of glycans from purified IgA proteins to generate monosaccharides. The released monosaccharides are then chemically modified into volatile compounds that are analyzed by gas-liquid chromatography.³¹ It was noted in the original description of the IgA1 hinge-region²² that the IgA1 hinge-region preparation contained carbohydrates; a follow-up study²⁴ clarified number of *O*-glycans and their sites of attachment in IgA1 myeloma proteins (Wal, Oso). The latter study combined approaches, including Edman degradation, gas-liquid chromatography, and mass spectrometry, to determine that a IgA1 myeloma protein (Oso) had *O*-glycans at five sites, S224, S228, S230, S238, and S240.²⁴ A comparative study of 11 IgA1 and 6 IgA2 myeloma proteins confirmed that IgA1 proteins generally contain *O*-glycans.²³ However, as detailed below, only two of these sites are commonly glycosylated on serum IgA1 of normal subjects.

A group of proteins called lectins have glycan-binding properties that render some to be useful probes for assessment of glycoproteins, depending of the specific saccharide or oligosaccharide recognized. For example, IgA1 *O*-glycan disaccharide GalNAc-Gal is recognized by jacalin, a lectin in jackfruit.^{42,43} Jacalin has been used for IgA1 isolation as well as probing of IgA1 *O*-glycans. Other lectins, including those in snails *Helix aspersa* and *H. pomatia*, have been used as probes for Gal deficiency of IgA1, as these lectins recognize terminal GalNAc.^{44–49} However, selection and testing of a candidate lectin must be done carefully and attention given to batch-to-batch variability, as lectin reactivity may depend on the geographical origin of the source of lectin (plants, snails).⁵⁰

Mass spectrometric approaches—The technological advances in biological mass spectrometry in the 1990s led to its use as the primary tool for analysis of IgA1 *O*-glycosylation. The sensitivity and molecular detail that can be inferred from glycopeptide chemical formulas enable quick assessment of the range of variably glycosylated forms of

IgA1 in a given sample. Both soft ionization techniques, matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), have been successfully applied to the analysis of IgA1 *O*-glycans. Two IgA1 hinge-region glycopeptides, containing four and five *O*-glycans, were identified by MALDI-TOF mass spectrometry.⁵¹ Follow-up analyses used normal serum IgA1,^{52,53} pooled serum of patients with IgAN,^{54,55} IgA1 isolated from pooled renal biopsies,⁶ and tonsillar IgA1.⁵⁶ Mass spectrometric analyses IgA1 hinge-region glycopeptides found less galactosylation in patients with IgAN than in healthy controls.^{6,54–56} IgA-specific proteases that released IgA1 hinge-region fragments of different lengths provided new tools for analysis of IgA1 *O*-glycosylation.^{27,50} These mass-spectrometry-based studies provided information on the range of differentially glycosylated forms of IgA1 and corroborated initial lectin-binding data. The results provided strong evidence that the composition of *O*-glycans of IgA1 from patients with IgAN distinctly differed from that of IgA1 from healthy controls. The next quest was to directly identify individual sites of *O*-glycosylation. That task was first accomplished by Renfrow et al. who applied a tandem mass spectrometry technique known as electron capture dissociation (ECD) that allowed sequencing of the IgA1 hinge-region glycopeptide with the *O*-glycosylated Ser and Thr residues remaining intact.⁵⁷ The non-ergodic method of ion fragmentation initiates an electron radical mechanism that causes the breakage of amino-acid backbone bonds without the loss of the labile *O*-glycans from the glycopeptide ions. Thus, individual sites of *O*-glycosylation can be directly identified in terms of the particular amino acid as well as site-specific *O*-glycan composition. These data identified Thr225, Thr228, Ser230, Ser232, and Thr236 as sites of glycan attachment in the IgA1 hinge-region *O*-glycoform with five *O*-glycans and Thr225, Thr228, Ser230, and Ser232 as the sites in two hinge-region *O*-glycoforms with four *O*-glycans. The ability to localize all sites of glycosylation in a single IgA1 preparation expanded the possibilities of defining the heterogeneity of different IgA1 preparations. Renfrow et al. pursued the *O*-glycan analysis of three distinct IgA1 myeloma proteins using reversed-phase liquid chromatography separation of tryptic hinge-region *O*-glycopeptides followed by ECD fragmentation, demonstrating the utility of high-resolution mass spectrometry.⁵⁸ Moreover, smaller fragments released by IgA1-specific proteases confirmed the findings.⁵⁸ In follow-up studies, all sites of *O*-glycosylation in the six most abundant IgA1 *O*-glycoforms of a separate IgA1 myeloma protein were fully localized.²⁷ The sites of attachment were consistent with earlier findings for another myeloma protein. The use of three distinct IgA1 hinge-region proteolytic fragments created a pattern of glycopeptide ion-abundance distributions in the mass spectra that demonstrated the possibility of quantifying individual IgA1 glycopeptide species.²⁷ In 2012, a new type of heterogeneity was identified, representing IgA1 *O*-glycopeptide isomers, *i.e.*, equal numbers of *O*-glycans in the hinge-region glycopeptide, but with different sites of attachment at Ser230, Thr233, and Thr236. These same sites were confirmed as the predominant sites of galactose deficiency in a myeloma protein as well as circulatory IgA1 isolated from two healthy individuals.⁵⁹ These two studies^{27,59} indicated that the sites of attachment likely originate from a semi-ordered synthesis of the clustered IgA1 *O*-glycans rather than through a random process or series of random attachments. These two studies also indicated that this level of molecular analysis could be achieved in a high-throughput fashion with liquid chromatography profiling of the range of differentially glycosylated IgA1 hinge-region glycopeptides combined with tandem mass-spectrometric analysis by electron transfer

dissociation (ETD). A multi-laboratory study of *O*-glycosylation analyses of IgA1 myeloma proteins⁶⁰ demonstrated that broader studies of IgA1 *O*-glycosylation heterogeneity could be undertaken with mass spectrometry. Therefore, well designed studies may identify specific *O*-glycosylated forms (*O*-glycoforms) of IgA1 as biomarkers for IgAN.

Macro-and micro-heterogeneity of IgA1 O-glycans—The clustered nature of IgA1 hinge-region *O*-glycans requires a unique series of considerations when defining the population of differentially *O*-glycosylated IgA1 molecules that may be present in an individual. First, there is the range of differently *O*-glycosylated hinge-region species. This overall heterogeneity, termed macroheterogeneity, is somewhat similar to the range of glycoforms present for *N*-glycosylated proteins, except that IgA1 hinge-region has multiple sites of *O*-glycan attachment that must be defined. The presence of isomers based on *O*-glycan site attachment further increases the difficulty of determining the macroheterogeneity of IgA1 *O*-glycosylation. In each IgA1 biological sample, the variability of *O*-glycans at a specific site (Thr225, Ser230, etc.) may be different and thus create a second level of heterogeneity, *i.e.*, site-specific microheterogeneity. Specifically, one question that can be addressed for any given population of IgA1 hinge-region molecules is: “How many times and at what level of abundance does a terminal GalNAc occur at Ser230?” Similar questions follow for each glycosylation site to define microheterogeneity in the context of macroheterogeneity, as shown in Figure 2.

The clustered nature of the *O*-glycan chains in the tandem octapeptide repeat of the IgA1 hinge region (Figure 1) requires a third level of heterogeneity definition, context. Adjacent glycans play a role in the local environment for a given site-specific chain. This clustered environment of adjacent glycans has been demonstrated to influence glycosyltransferase activity. Sialylation of an adjacent site can influence the galactosylation of a site of interest, thus leading to a site that has only terminal GalNAc. Conversely, recognition of specific glycan structures by lectins or antibodies at a given site can be blocked by the steric hindrance of *O*-glycans at adjacent sites.⁶¹ The clustered *O*-glycans of the IgA1 hinge region are not individual modifications to the protein but a series of inter-related and interacting modifications that create different glycan surfaces for interaction with other biological molecules. Thus, the answer to the question of how often a given site of *O*-glycosylation has only terminal GalNAc is incomplete without the context of *what O*-glycans are immediately adjacent to the terminal GalNAc.⁶²

Abnormality of IgA1 O-glycosylation in IgAN (galactose-deficient IgA1)

A number of experimental approaches to the study of IgA1 *O*-glycosylation in IgAN have been used: lectin binding to the *O*-glycans *in situ* on IgA1;^{44,47,63–66} chromatographic separation of the released *O*-glycans, both in their intact form and as free monosaccharides,^{44,67–69} and, more recently, mass spectrometry of the isolated IgA1 hinge region complete with its *O*-glycans.^{27,57–61} Uniformly, these studies have demonstrated an increased proportion of Gal-deficient IgA1 *O*-glycoforms in the serum in IgAN, a finding that has been consistently reproduced in populations of different ethnic and geographic origin. While the early reports of defective galactosylation of IgA1 *O*-glycans in IgAN were focused on studies of serum IgA,^{44,47,63,67,70} later studies of IgA1 eluted from isolated glomeruli have

identified the same *O*-galactosylation abnormalities in mesangial IgA1, strongly implicating altered IgA1 *O*-galactosylation in the mechanism of IgA deposition in IgAN.^{5,6} Intriguingly, the presence of Gal-deficient IgA-containing immune complexes has also been reported in the urine of patients with IgAN but not in patients with non-IgAN proteinuric glomerular disease.⁷¹

These observations raise an obvious question: is defective *O*-galactosylation common to all serum proteins in IgAN? Of the few serum proteins which are *O*-glycosylated, two have been studied in detail: C1 inhibitor (C1inh) and IgD. In both instances, there was no alteration of the *O*-glycosylation profile of either C1inh or IgD in IgAN.^{47,72} These observations argue against a generic defect of *O*-glycosylation of serum proteins in IgAN and suggest that any abnormality is likely to be B cell-specific.

The pattern of IgD *O*-glycosylation is normal in patients with IgAN. This finding implies that the changes in IgA1 *O*-glycosylation are not caused by defective expression or function of glycosylating enzymes affecting the entire B-cell lineage in IgAN. Rather, it is limited to changes in the action of glycosylating enzymes at the time of antigen-induced B-cell activation and class switching that affects *O*-glycosylation of IgA1 in IgAN. Furthermore, this does not appear to be a general IgA1 class-switched B cell phenomenon. IgA1 *O*-glycosylation profiles appear to be heavily influenced by the site of antigen encounter. IgA1 generated after an encounter with an antigen at a mucosal surface (*e.g.*, *Helicobacter pylori*)⁷³ is strikingly and significantly undergalactosylated, whereas IgA1 generated after antigen encounter within the systemic compartment (*e.g.*, tetanus toxoid [TT]) is relatively heavily galactosylated. Patterns of *O*-glycosylation in anti-*H. pylori* and anti-TT IgA1 antibodies are identical in healthy subjects and patients with IgAN, suggesting that differential *O*-glycosylation of IgA1 antibodies after antigen exposure at different induction sites is a normal process.

Supporting the concept that IgA1 *O*-glycosylation can be differentially controlled within IgA1-committed B cell subsets, there is evidence from *in vitro* studies and animal models that changes in the microenvironment modify IgA1 *O*-glycosylation. Most notably, this influence is mediated by T-helper 2 cytokines and interleukin-6 which down-regulate key glycosylating enzymes and lead to the generation of aberrantly glycosylated IgA1.^{74–78} There is also emerging evidence for epigenetic control of IgA1 *O*-glycosylation in IgAN. MicroRNAs (miRNA) are endogenous, short, noncoding single-stranded RNA molecules that regulate gene expression. Up-regulation of a specific miRNA (miR-148b) in peripheral blood mononuclear cells has been associated with decreased expression of core 1 β 1,3-galactosyltransferase (C1GalT1) and production of Gal-deficient IgA1.⁷⁹ In the same study, over-expression of miR-148b in a surface IgA1-positive human B-cell lymphoma cell line resulted in down-regulation of *C1GALT1* mRNA levels and a corresponding increase in production of Gal-deficient IgA1.

Two recently published QT-GWAS (quantitative trait-genome wide association studies), identified clear associations between SNPs (single nucleotide polymorphisms) in the noncoding region of *C1GALT1*, the gene encoding the C1GalT1 galactosyltransferase, and serum levels of Gal-deficient IgA1.^{21,80} This observation is entirely consistent with the

concept that changes in IgA1 *O*-glycosylation in IgAN are secondary to maturation-dependent transcriptional regulation of the *CIGALT1* gene, likely the result of sequence variation in the un-translated region of its mRNA or in promoter or enhancer regions that affect DNA methylation state or transcription factor binding. One strongly associated SNP, rs758263, lies within the core promoter region for *CIGALT1*, although it is not within the previously identified transcription factor-binding sites at this locus.⁸¹ *In silico* analyses identified several other strongly associated SNPs that lie within consensus transcription factor-binding elements of the gene. Intriguingly, a binding site for miR-148b has also been identified within the *CIGALT1* risk haplotype (1365G>A polymorphism or rs1047763) reinforcing the potential role of miRNAs in control of IgA1 *O*-glycosylation in IgAN.

These two recent QT-GWA studies also provide, for the first time, a genetic explanation for the previously reported heritability of IgA1 *O*-glycosylation. Studies of familial and sporadic IgAN cohorts have previously estimated the heritability of Gal-deficient IgA1 to be between 54% and 76% and, in a recent study of healthy monozygotic and dizygotic twin pairs using the classic twin model, the heritability of Gal-deficient IgA1 was found to be as high as 80%.^{82–86} Consistent with these observations, the reported SNP associations were not restricted to IgAN but were also found in healthy subjects and cases of membranous nephropathy, in both white and Chinese populations.

One unexpected finding from these QT-GWA studies was the observation that, despite the high incidence and poorer outcomes of IgAN in China, levels of Gal-deficient IgA1 in Chinese IgAN cases were lower than in white IgAN cases and indeed were comparable to those seen in a healthy white population, and this corresponded with the low frequency of the identified *CIGALT1* risk haplotype in the Chinese population. This observation raises important and, as yet, unanswered questions concerning the pathogenic importance of changes in IgA1 *O*-galactosylation in different ethnic populations.

In common with the *O*-glycosylation defect of IgA1 there are a number of other features characteristic of the IgA immune response in IgAN. These include persistent systemic overproduction of polymeric IgA1 against a variety of systemic and mucosal antigens,^{87–91} failure of affinity maturation of serum IgA1 antibodies⁹² and systemic IgA1 immune responses that tend to be exaggerated and prolonged.⁹³ These features are also characteristic of a mucosal IgA immune response.^{73,94,95} While it is known that mucosal polymeric IgA1-producing plasma cell numbers^{96,97} and mucosally secreted IgA antibody responses^{98,99} are reduced in IgAN, it is clear that the *site* of antigen encounter heavily influences the ultimate phenotype of both T and B lymphocytes, both through their subsequent expression of cell-surface homing receptors and their functional capabilities, independent of their final site of residence.¹⁰⁰ It seems reasonable to assume, therefore, that the mucosa may well be an ideal microenvironment for the programming of naïve B cells to develop into plasma cells capable of secreting the polymeric, low affinity and Gal-deficient IgA1 that ultimately forms the substrate for IgA immune complex-formation in IgAN, even if it is not their eventual site of residence. Intriguingly, in addition to the cytokine milieu of the mucosa, the gut microbiome itself may contribute to this programming. Toll-like receptors (TLR) play key roles in innate immunity to microbial pathogens *via* recognition of a diverse range of pathogen-associated molecular patterns, such as bacterial lipopolysaccharide (LPS), RNAs, and DNAs.¹⁰¹ TLR-9

is expressed in the endoplasmic reticulum of B lymphocytes and binds bacterial DNA sequences with un-methylated cytosine-guanine dinucleotide motifs.¹⁰² Ligation of TLR-9 leads to polyclonal activation of B cells, class switching, and immunoglobulin production. IgA secretion by mucosal lamina propria cells is increased after TLR-9 stimulation, implying that mucosal B cells can recognize pathogen-associated molecular patterns and secrete IgA in a T-cell-independent manner.^{103,104} Perhaps more significant is the observation that ligation of B-cell TLR-4 by bacterial LPS induces methylation of the *COSMC* gene, leading to reduced activity of C1GalT1 and under-galactosylation of IgA1.¹⁰⁵

Further elucidation of the biochemical pathways driving maturation-dependent transcriptional regulation of the *C1GALT1* gene will undoubtedly help us understand the driving forces for Gal-deficient IgA1 production in IgAN. This knowledge will aid in potential identification of new therapeutic targets in IgAN.

IgA1-producing cells in IgAN and O-glycosylation pathways

As stated earlier, IgA1 has 3–6 core 1 O-glycans, *i.e.*, containing Ser/Thr-linked GalNAc with a β 1,3-linked Gal. These glycans may be mono- or di-sialylated. O-glycosylation of IgA1 occurs in the Golgi apparatus of IgA1-secreting cells, as a stepwise post-translational modification that, unlike translation, is not a template-driven process. Instead, it depends on the arrangement of glycosyltransferases in organized enzyme complexes that control the sequential addition of monosaccharides to the acceptor (glyco)protein during its transition through Golgi system. Notably, O-glycans in the hinge region of IgA1 are not required for folding and export of the immunoglobulin.¹⁰⁶

IgA1 O-glycosylation begins with attachment of GalNAc to some of the Ser/Thr residues in the hinge region. Members of a family of 20 enzymes, *N*-acetylgalactosaminyl-transferases (GalNAc-Ts), catalyze this process (Figure 3). It has been proposed that GalNAc-T2 is the main GalNAc-T enzyme responsible for the initiation of O-glycosylation of IgA1,^{26,107} but apparently other GalNAc-Ts may contribute to the process, including GalNAc-T1, -T11, and -T14.^{108,109} GalNAc-Ts determine not only the sites of GalNAc attachment but also the final glycan density.

The attached GalNAc residues can be then modified by addition of Gal, mediated by core 1 β 1,3-galactosyltransferase (C1GalT1).¹¹⁰ Notably, the production of the active enzyme depends on its chaperone (C1GalT1C1, Cosmc).^{111–113}

The core 1 structures of IgA1 may be further modified by sialyltransferases that attach sialic acid to Gal (mediated by an ST3Gal enzyme) and/or GalNAc residues. Sialylation of GalNAc is mediated by ST6GalNAc-II, as the usual ST6GalNAc-I is not expressed in IgA-producing cells (Figure 3).^{109,114,115}

It is difficult to obtain IgA1-producing cells in large numbers from human subjects, whether from bone marrow or mucosal tissues. However, there are IgA1-producing cells in peripheral blood, although the number of such cells is very low and does not allow direct biochemical studies of IgA1 O-glycosylation. To address that issue, we have developed a new model,

immortalized IgA1-secreting cells derived from the cells in peripheral blood of patients with IgAN, and healthy and disease controls. These IgA1-producing cells thus provided a new tool for studies of normal and aberrant *O*-glycosylation of IgA1.¹¹⁶ We found that IgA1 secreted by the cells from patients with IgAN had higher degrees of Gal deficiency of the *O*-glycans than that from cells of healthy controls. In addition, relative degree of Gal deficiency of the IgA1 secreted by IgA1-producing cells correlated with that of serum IgA1 from the corresponding donors.¹¹⁶ These findings indicated that the immortalization process likely has not altered the *O*-glycosylation machinery of the IgA1-producing cell lines. We have therefore used these cells as a model to study expression and activity of key enzymes involved in IgA1 *O*-glycosylation.

Comparative studies revealed that polymeric IgA1 is the molecular form most affected by Gal deficiency. Moreover, we determined that production of Gal-deficient IgA1 was related to decreased expression of *C1GALT1* and *C1GALT1C1* and elevated expression of *ST6GALNAC2*.¹¹⁶ These conclusions were confirmed by assessing enzyme activities of C1GalT1 and ST6GalNAc-II and by using *C1GALT1*-, *C1GALT1C1*-, and *ST6GALNAC2*-specific siRNA knock-down with subsequent biochemical analyses.^{21,61,77,115} Thus, multiple mechanisms impact glycosylation of IgA1 and a reduced Gal content originates from abnormal expression and activity of several key glycosyltransferases in IgA1-producing cells.¹¹⁶ Moreover, the serum level of Gal-deficient IgA1 is genetically co-determined^{12,18,19,21,84,85,117} and may be altered by environmental factors and cytokines.^{77,78}

Using a panel of cloned IgA1-secreting cells, we have demonstrated direct effects of several cytokines, such as IL-6, on production of Gal-deficient IgA1. In cell culture, IL-6 supplementation enhanced production of total IgA1 by cells from healthy controls and patients with IgAN. However, only cells from patients with IgAN had elevated production of Gal-deficient IgA1 after IL-6 supplementation, indicating that the cells from healthy controls and IgAN patients responded differently to IL-6.⁷⁷ IL-6 dysregulated expression and activities of several key glycosyltransferases in IgA1-secreting cells from IgAN patients compared to those from healthy controls. Specifically, IL-6 further reduced expression C1GalT1 and its chaperone Cosmc and increased expression and activity of ST6GalNAc-II in IgA1-secreting cells from IgAN patients.¹¹⁶ IL-6 activity is mediated by IL-6 receptor/gp130 complex that activates JAK/STAT signaling pathway. Detailed analyses of IgA1-secreting cells from IgAN patients revealed an aberrant regulation of IL-6-induced JAK/STAT signaling, leading to an enhanced and persistent phosphorylation of STAT3.⁷⁸ This IL-6-mediated overproduction of Gal-deficient IgA1 can be blocked by small-molecule inhibitors of JAK/STAT signaling.⁷⁸

Other factors involved in B cell/plasma cell differentiation and survival that may be relevant to IgAN include B-cell activating factor (BAFF) and APRIL.^{18,19,118} Notably, over-expression of human BAFF in mice leads to overproduction of IgA and development of IgA glomerular deposits.¹¹⁹

These observations illustrate a possible association between the clinical onset and flares in IgAN activity that often coincide with an upper-respiratory-tract infection. Perhaps these

environmental factors (*i.e.*, mucosal infections) induce production of cytokines (*e.g.*, IL-6) and growth factors (*e.g.*, BAFF, APRIL) that subsequently up-regulate aberrant *O*-glycosylation of IgA1 and/or support survival of Gal-deficient IgA1-secreting cells in susceptible individuals.^{12,117,118} Some of these aberrant responses can be mitigated in selected IgAN patients by blockade of specific signaling pathways, *e.g.*, targeting a protein-tyrosine kinase receptor(s).⁷⁸

IgA1 *O*-glycosylation and interaction with IgA receptors in IgAN

Currently, four different classes of IgA receptors have been characterized in detail: the hepatic asialoglycoprotein receptor (ASGP-R) that recognizes the asialo-glycan moieties of many glycoproteins, including IgA and is expressed mainly in the liver;^{120–122} the polymeric immunoglobulin receptor that binds J-chain-containing immunoglobulins (IgA and IgM) and is found predominantly in the mucosae;^{123,124} FcαR1 (CD89) that is specific for IgA through binding the constant region of the heavy chain of IgA1 and IgA2 and is expressed by cells of myeloid lineage,^{125,126} and the transferrin receptor (CD71) that is a multi-ligand receptor expressed ubiquitously by proliferating cells, including mesangial cells.^{127–129} There is a significant body of experimental evidence showing that binding of IgA to the ASGP-R, CD89, and CD71 is heavily influenced by changes in the *O*-glycan composition of the IgA1 hinge region and that this feature is likely to have direct consequences on the systemic handling of IgA and intrarenal responses to IgA deposition in IgAN.

There are other IgA receptors on circulating myeloid cells and in the kidney. DC-SIGN (CD209), a C-type lectin receptor on the surface of both macrophages and dendritic cells, binds secretory IgA.^{130,131} Within the kidney, an Fc α/μ receptor,¹³² an integrin IgA receptor,¹³³ and β-1,4-galactosyltransferase 1¹³⁴ all bind IgA, although little is known about their biology, the influence of IgA1 *O*-glycans on receptor binding, and their role in IgAN.

IgA is normally cleared from the circulation by the liver and leucocytes and this process is impaired in IgAN,¹³⁵ leading to persistence of IgA immune complexes in the circulation that is likely to lead to mesangial IgA deposition. This conclusion is consistent with the earlier observation that anti-allotype monoclonal antibody IgG inhibits the hepatobiliary transport of polymeric IgA.¹³⁶ The slowed clearance is due to a combination of hepatic ASGP-R down-regulation (a key feature in secondary IgAN due to cirrhosis), limited access to the ASGP-R *via* the space of Disse due to the large size of IgA immune complexes in IgAN (driven by IgG/IgA anti-IgA *O*-glycan autoantibodies) and, perhaps, decreased ASGP-R binding as a result of reduced *O*-galactosylation of the IgA1 hinge region.^{44,137–140} In addition, down-regulation of myeloid CD89 expression, reduced CD89-mediated IgA endocytosis and impaired binding of Gal-deficient IgA to CD89 in IgAN (and alcoholic cirrhosis)^{141–143} also contribute to delayed IgA clearance.

Gal-deficient IgA immune complexes have also been reported to induce cleavage and shedding of the extracellular domain of membrane-bound CD89, resulting in formation of high-molecular-mass IgA-soluble CD89 (IgA-sCD89) complexes that are prone to mesangial deposition.¹⁴⁴ Murine studies suggest that activation of mesangial cells by IgA immune complexes requires sCD89, a process that is also dependent on tissue transglutaminase 2.¹⁴⁵ Recurrent IgAN following transplantation also appears to be

associated with higher levels of these IgA-sCD89 complexes.¹⁴⁶ However, conflicting data suggest that, in humans, IgA-sCD89 complexes may in fact be an epiphenomenon and neither specific nor relevant to the development of IgAN.^{147,148}

It has been recognized for many years that mesangial cells bind IgA in an *O*-glycan-dependent manner. This binding is important for both intraglomerular clearance of IgA deposits through receptor-mediated endocytosis and for triggering mesangial-cell activation with the consequent release of pro-inflammatory and pro-fibrotic mediators that drive the glomerulonephritis in IgAN.^{149–151} CD71 is the best characterized mesangial-cell IgA receptor. It is overexpressed on the surfaces of proliferating mesangial cells in IgAN, co-localizes in renal biopsies with IgA immune deposits. Upon binding IgA immune complexes, in concert with tissue transglutaminase 2, mesangial-cell proliferation and secretion of soluble mediators, including IL-6 and TGF- β , ensue.^{128,129,152} Significantly, the binding of Gal-deficient IgA to CD71 appears to further enhance the expression of CD71 on proliferating mesangial cells, creating an autoamplification loop for self-perpetuating glomerular injury.¹²⁷

There is emerging evidence that IgA also binds to podocytes¹⁵³ and proximal tubule epithelial cells (PTEC),^{154,155} both of which are likely to be bathed in filtered Gal-deficient IgA1 complexes.^{71,156} It is not unreasonable, therefore, to speculate that Gal-deficient-IgA1 complexes may also accentuate podocyte and PTEC injury in IgAN and this may in part explain the glomerulosclerosis and tubulointerstitial inflammation and fibrosis that occur in more severe forms of IgAN.

Multi-hit hypothesis and autoantibodies specific for Gal-deficient IgA1

IgAN damages kidneys as innocent bystanders, as indicated by frequent recurrence of disease in renal allografts^{157,158} and the clearance of immune deposits in a kidney transplanted from a donor with subclinical IgAN into a patient with non-IgAN renal disease within several weeks.¹⁵⁹ There is considerable evidence that the mesangial immune deposits in IgAN originate from circulating IgA1-containing immune complexes, including (1) patients with IgAN have elevated blood levels of IgA and IgA1 immune complexes;¹⁶⁰ (2) IgA in the circulating immune complexes and mesangial deposits share idiotypic determinants, although a disease-specific idio type has not been identified;¹⁶¹ and (3) IgA1 in the circulating immune complexes^{46,66} and in the renal deposits^{5,6} is Gal-deficient compared to serum IgA1 in healthy individuals.

We have proposed a four-hit pathogenic autoimmune process leads to the development of primary IgAN (Figure 4): Gal-deficient IgA1 in the circulation (hit #1) is recognized as autoantigen by circulating autoantibody (IgG or IgA) specific for terminal GalNAc on Gal-deficient *O*-glycans in the hinge region of IgA1 (hit #2) to form immune complexes (hit #3) that deposit in the kidney to stimulate mesangial cells (hit #4).¹⁶² In many patients, activation of the complement cascade ensues and mesangial cells proliferate and overproduce components of extracellular matrix, cytokines, and chemokines.¹³⁹ Some cytokines directly cause podocyte injury to induce proteinuria.^{163,164} The steps in the process of glomerular injury are likely modulated by environmental and genetically

determined factors, such as complement regulation^{12,20,117,165} and *O*-glycosylation of IgA1.^{21,80,166}

Other hypotheses have been proposed to explain the pathogenesis of IgAN. One postulates that uncomplexed galactose-deficient IgA1 deposits in the mesangium without significant initial kidney damage. At a later time, circulating glycan-specific antibodies pass through fenestrae in the glomerular capillaries to enter the mesangium to bind the IgA1 *in situ*, initiating glomerular injury.¹⁶⁷ Another hypothesis proposes that exposure to gluten increases the circulating burden of immune complexes with Gal-deficient IgA1 bound to soluble CD89.¹⁶⁸ As stated above, the pathophysiological significance of these CD89-containing complexes in primary IgAN is unclear.

The type and location of immune proteins in the glomerulus affect the renal outcomes in patients with IgAN. Co-deposition of IgG with IgA in the mesangium has been associated with a worse renal survival.^{169–172} In contrast, a recent study found that IgG co-deposition and the presence of immune proteins within the peripheral capillary walls each associated with greater histological activity (crescents or segmental glomerular necrosis) but only the latter was associated with a poor renal outcome (renal replacement therapy, transplantation, death, or doubling of serum creatinine concentration).¹⁷³ An adverse impact of immune deposits in the peripheral capillary loops on renal clearance function is consistent with prior studies about three decades ago.^{174–177} In a more recent study, 38 of 190 Spanish patients IgAN with preserved renal clearance function (estimated glomerular filtration rate >80 mL/min/1.73 m²) at the time of biopsy exhibited mesangial C4d. This finding was associated with greater proteinuria at diagnosis, and more nephritic flares, greater proteinuria, larger reduction in estimated glomerular filtration rate, and worse kidney survival over the next 15 years.¹⁷⁸ The mechanism of complement activation to generate this complement fragment is not yet clear.

About half of the kidney biopsies from patients with IgAN do not have mesangial IgG detected by routine immunofluorescence microscopy. It is possible that this finding is due to the presence of IgA rather than IgG anti-glycan autoantibodies in the immune complexes bound to Gal-deficient IgA1 or to a low sensitivity for detection of IgG. To address the latter possibility, we retrieved remnant frozen renal biopsy tissues from five IgAN patients that had mesangial IgG on routine immunofluorescence microscopy, 10 IgAN patients whose biopsies did not, and one patient with primary membranous nephropathy with IgG staining.⁸ IgG-IgA glomerular co-localization was confirmed by confocal microscopy. We processed the pooled biopsy tissues to extract glomerular IgG. The IgG from the patients with IgAN displayed autoantibody characteristics as shown by the capacity to bind to Gal-deficient IgA1.¹⁷⁹ IgG was also recovered from the 10 pooled biopsy specimens that did not exhibit IgG by routine immunofluorescence microscopy. Furthermore, it also bound Gal-deficient IgA1, in contrast, IgG from the membranous nephropathy biopsy tissue did not. These results support the significance of IgG specific for Gal-deficient IgA1 in the pathogenesis of IgAN.

Recent studies have shown the impact of Gal-deficient IgA1 and the corresponding autoantibodies on the clinical course of IgAN. The serum level of Gal-deficient IgA1 is

associated with the activity of IgAN, as manifested by microscopic hematuria or proteinuria^{180,181} as well as development of end-stage renal disease.^{180,182} Serum levels of IgG autoantibodies specific for Gal-deficient IgA1 also correlate with disease severity, as assessed by magnitude of proteinuria,¹⁷⁹ histological prognostic group,¹⁸³ and renal survival.¹⁸⁴ Thus, the serum level of these IgG autoantibodies has shown potential as a biomarker for the clinical severity of IgAN. However, these serum biomarkers have not yet proven sufficient to identify patients IgAN well enough to forego a kidney biopsy and are not accurate enough for prediction of long-term outcomes for individual patients.

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Hinge-region amino acid sequence

²²²VPST*PPT*PS*PS*T*PPT*PSPSC²⁴¹

Figure 1. Structure and O-glycosylation of human circulatory IgA1.

Modeled structure of glycosylated monomeric IgA1 based on PDB ID: 1IGA (top) and hinge-region amino acid sequence with attachment sites of six O-glycans (bottom). Modeled O- and N-glycans are depicted as spheres for clarity and are based on observed glycoforms: red for Gal-GalNAc; orange for GalNAc; and magenta for N-glycan (NeuAc)₂(Gal)₂(GlcNAc)₂+(Fuc)₁(Man)₃(GlcNAc)₂. Hinge-region non-glycosylated amino-acid residues are in blue. There are up to six sites with O-glycans in the IgA1 hinge region: at T225, T228, S230, S232, T233, and T236 (marked by stars in the bottom amino-acid sequence). Model was generated by Tyler Stewart; figure is reproduced with permission.¹⁸⁵

Site-specific microheterogeneity of desialylated IgA1 hinge region O-glycans						
	225T	228T	230S	232S	233T	236T
No glycan	<1 %	N.D.	1/10	N.D.	> 1/2	>1/2
GalNAc only	<1 %	1 %	1/3	< 1%	< 1/4	1/4
Gal-GalNAc	Predominant	Predominant	1/2	Predominant	1/4	< 1/4

	T 225	T 228	S 230	S 232	T 233	T 236

Figure 2. Site-specific microheterogeneity of normal human serum IgA1 hinge-region O-glycans.

This figure summarizes results based on relative distributions of desialylated O-glycopeptides observed by high-resolution mass spectrometry.⁵⁹ This distribution includes the delineation of amino-acid positional isomers identified by liquid chromatography-mass spectrometry and ECD-tandem mass spectrometry.⁵⁹ S230, T233 and T236 are the primary sites of Gal deficiency and variable positional isomers in normal serum IgA1. S225, S228, and S232 are predominantly occupied by Gal-GalNAc disaccharide; these sites are rarely non-glycosylated (N.G.) or occupied by Gal-deficient species. Figure is reproduced with permission.¹⁸⁶

Figure 3. Biosynthetic pathways of IgA1 O-glycosylation.

O-glycans are synthesized in a step-wise manner, beginning with the attachment of *N*-acetylgalactosamine (GalNAc) to the oxygen atom of serine (Ser) or threonine (Thr) that is catalyzed by GalNAc-transferases. This step can be followed by addition of galactose (Gal) (catalyzed by C1GalT1; chaperone Cosmc is required for production of active C1GalT1 enzyme). Sialic acid (*N*-acetylneuraminic acid) can be added to each glycan by different enzymes, ST3Gal-I for sialylation of Gal and ST6GalNAc-II for sialylation of GalNAc. Sialylation of terminal GalNAc (*i.e.*, without Gal) prevents subsequent addition of Gal. Pathway is proposed based on published primary data and reviews^{77,109,115,116,162,185–190}. Color coding is per Symbol Nomenclature for Glycans: yellow-filled square, GalNAc; yellow-filled circle, Gal; purple-filled diamond, *N*-acetylneuraminic acid.

Figure 4. A multi-hit hypothesis of IgAN pathogenesis.

This model assumes formation of immune complexes in the circulation and their subsequent mesangial deposition. An alternative hypothesis assumes that aberrantly glycosylated IgA1 deposits in the mesangium as lanthan deposits that are later bound by newly appearing autoantibodies, resulting in the *in situ* formation of immune complexes.¹⁶⁷ *Hit 1:* Increased production of Gal-deficient IgA1 by a sub-population of IgA1-secreting cells.¹¹⁶ *Hit 2:* Formation of autoantibodies with specific characteristics of the variable region of the heavy chain that recognize Gal-deficient IgA1.¹⁹¹ *Hit 3:* Formation of pathogenic immune

complexes from auto-antigen (Gal-deficient IgA1) and Gal-deficient IgA1-specific autoantibody.^{150,192–194} *Hit 4:* Deposition of pathogenic immune complexes in the mesangium, activation of mesangial cells, and induction of glomerular injury.^{127,150,163,164,192–196} These hits may be regulated by distinct genetic loci, identified by genome-wide association studies (GWAS) as associated with risk for IgAN as well as by other loci (for review see^{12,117}). An alternative hypothesis assumes that aberrantly glycosylated IgA1 deposits in the mesangium as lathane deposits that are later bound by newly appearing autoantibodies, resulting in the *in situ* formation of immune complexes.¹⁶⁶